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Research Article

Effect of Salicylic Acid Foliar Application on Bioactive Compounds and Antioxidant Activity in Holy Basil (*Ocimum sanctum* L.)

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Holy basil (*Ocimum sanctum* L.) has been used extensively in Thai traditional medicine, where it is commonly utilized as a part of herbal remedies for treating various ailments. Cultivation methods using exogenous salicylic acid (SA) to induce secondary metabolites have been documented in various plant species. Nevertheless, there is no reported information available on holy basil. Thus, the present study aimed to investigate the impact of SA foliar application on the bioactive compounds and antioxidant activity of holy basil. SA at concentrations of 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 mM was foliar sprayed 30 days after transplanting (DAT) compared to spraying with tap water as the control. The plants were harvested at 33 DAT. Exogenous SA at 0.1–1.5 mM enhanced the contents of bioactive compounds and improved antioxidant activity. The highest contents of eugenol (17,829.53 ± 243.11 μg/g dry extract), total phenolics (444.10 ± 2.80 mg GAE/g dry extract), and total flavonoids (382.69 ± 6.49 mg QE/g dry extract) were achieved at 1.0 mM SA foliar application, which was 282.96, 1.76, and 2.14 times, respectively, over control. Furthermore, the greatest antioxidant activity was observed in the 1.0 mM SA treatment. In contrast, the 2.0 and 2.5 mM SA treatments had lower levels of antioxidant activity and bioactive compounds than the control. The results of this study suggest that exogenous 1.0 mM SA foliar application is an effective method to produce enriched bioactivity in holy basil.

1. Introduction

Holy basil (*Ocimum sanctum* L.) is one of the most popular medicinal plants in the Lamiaceae family. Since ancient times, this plant species has been extensively employed in traditional medicine to prevent and treat ailments [1]. Its leaves are rich in eugenol, methyl eugenol, phenolics, flavonoids, terpenoids, neolignans, and fatty acid derivatives [2]. These constituents possess pharmacological properties that modulate various biological activities, including antioxidant, anticancer, antiasthmatic, anti-inflammatory, antiallergic, antidiabetic, and antistress [1–3]. In Thai traditional medicine, holy basil leaves are used in herbal remedies for treating cancer as well as relieving flatulence and asthma.

Components derived from plants, such as roots, shoots, leaves, flowers, fruits, and seeds, play a crucial role as fundamental elements in creating herbal products. The high content of bioactive compounds in medicinal plants is regarded as a high-quality raw material for the herbal drug industry. The accumulation of secondary metabolites in these plants can be induced by several cultivation methods [4]. Biotic elicitors are commonly applied to enhance the synthesis and accumulation of bioactive compounds in

plants since it is convenient, fast, safe, eco-friendly, and quick strategy [5]. A highly effective biotic elicitor known as salicylic acid (SA) or 2-hydroxybenzoic acid causes a variety of physiological and developmental responses in plants, including seed germination [5, 6], stomatal movement [5, 6], pigment accumulation [5, 7], photosynthesis [5, 6, 8], ethylene biosynthesis [5], enzyme activities [5], nutrient uptake [5, 8], flower induction [5, 6], and membrane functions [5]. Furthermore, SA has been increasingly recognized for its role in enhancing plant tolerance to various abiotic stresses such as salinity, drought, and high temperature [9]. Exogenous SA has been applied to increase bioactive compounds in many plant species such as sweet basil [10–12], peppermint [8, 13], marjoram [10], amaranth [14], and *Ammi visnaga* [15]. The efficiency of exogenous SA to induce secondary metabolites depends on many factors. SA concentration is one of the important factors influencing bioactive compounds in plant species [16]. Low SA concentration might be not enough for physiological process activation or to regulate gene expression for secondary metabolite accumulation [17]. In contrast, high SA concentration usually causes deleterious effects. However, the optimal SA concentration to induce bioactive compounds in each plant species tends to be specific, like 0.1 mM SA for sweet basil [12], 0.01 mM SA for amaranth [14], 0.5 mM SA for peppermint [8], and 1 and 2 mM for *Ammi visnaga* under water shortage and normal irrigation, respectively [15]. These values indicate that each plant species shows different responses to SA doses. However, no information has been reported on holy basil. Therefore, the purpose of this work was to examine the eugenol content as well as the total phenolic, flavonoid, and antioxidant activity in holy basil at various exogenous SA application concentrations. The findings of this study will provide technical guidance for the production of enriched bioactivity in holy basil.

2. Materials and Methods

2.1. Plant Cultivation, Salicylic Acid Treatment, and Plant Growth Measurement. Holy basil, purple-type seeds of OS18 were collected at Thammasat University, Thailand. The seeds were sown into 105-cell trays containing peat moss as substrate. The 30-day-old seedlings were transplanted into 8×15-inch planting bags. The physical and chemical properties of the commercial substrates, as described by Rithichai et al. [18], were utilised in this study. The base fertilizer consisted of 30 g/plant of manual fertilizer and 0.65 g/plant of 16-16-16 (N-P-K). After transplanting, the plants were fertilised with a solution containing 6.5 g/L urea at a rate of 100 ml/plant for 7 days. At 15 days after transplanting (DAT), the plants were further fertilised with 0.65 g/plant of 16-16-16. Water was irrigated daily to maintain soil moisture. The plants were cultivated in a greenhouse located at Thammasat University, Rangsit Campus, Pathum Thani, Thailand. Plants at 30 DAT were foliar sprayed with SA at the concentrations of 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 mM for 100 mL/plant. The control was sprayed with tap water. Tween 20 at 0.1 mL/L was used to help spread and keep the spray solution on the foliage. Plant height was measured at 33 DAT, then the plants were cut at the soil surface, and shoot fresh weight was recorded. Mature leaves were separated, and leaf fresh weight was collected. Leaves were dried by freeze drier, and leaf dry weight was determined. The freeze-dried samples were ground and kept at −20°C for further use.

2.2. Preparation of Alcoholic Extracts. Plant extract samples were prepared as described by Rithichai et al. [18]. After calculating the dry extract's yield, samples of the dried extract were stored at −20°C for later use.

2.3. Determination of Eugenol Content. Eugenol content was conducted following the method described by Autaijamsripon et al. [19] with some modifications. Ten milligrams of the extract were dissolved with 1 mL of HPLC methanol, and the solution was sonicated for 20 min. Each sample was filtered through a 0.22 μm filter membrane. To determine the eugenol content, ultrahigh performance liquid chromatography (UHPLC) was used. The Nova-Pak C18 column (150×3.9 mm i.d., 4 μm) with a guard column was connected to the Shimadzu Nexera LC-30 A with isocratic elution mode. Ten microliters of the sample were injected for 4 min. Acetonitrile, water, and methanol were combined in the mobile phase in a ratio of 50 : 40 : 10. There was a 0.7 ml/min flow. The wavelength of the detector was tuned to 280 nm (Figure 1). Eugenol content was calculated using a lab solution program and expressed as μg/g dry extract.

2.4. Determination of Total Phenolic and Flavonoid Contents. Total phenolic and flavonoid contents were determined using the Folin–Ciocalteu colourimetric method [18] and aluminium chloride colourimetric assays [20], respectively. To examine the total phenolic and flavonoid contents, a microplate reader (Power Wave XS, BioTek) was utilised to measure the absorbance at wavelengths of 765 and 510 nm, respectively. Total phenolic content was expressed as milligram gallic acid equivalent per gram dry extract (mg GAE/g dry extract), while the total flavonoid content was expressed as milligram quercetin equivalent per gram dry extract (mg QE/g dry extract).

2.5. Determination of Antioxidant Activity. Antioxidant activity was measured using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, employing a method adapted from Rithichai et al. [18]. Absorbance was measured at 520 nm using a microplate reader. EC_{50} values, which represent the concentration of the sample needed to inhibit 50% of the DPPH free radical, were calculated using a regression equation. The positive control was BHT (butylated hydroxytoluene).

2.6. Statistical Analysis. The experiment employed a completely randomized design. Data were subjected to analysis of variance (ANOVA) using SAS software. Differences between means were achieved by Tukey's Honestly Significant

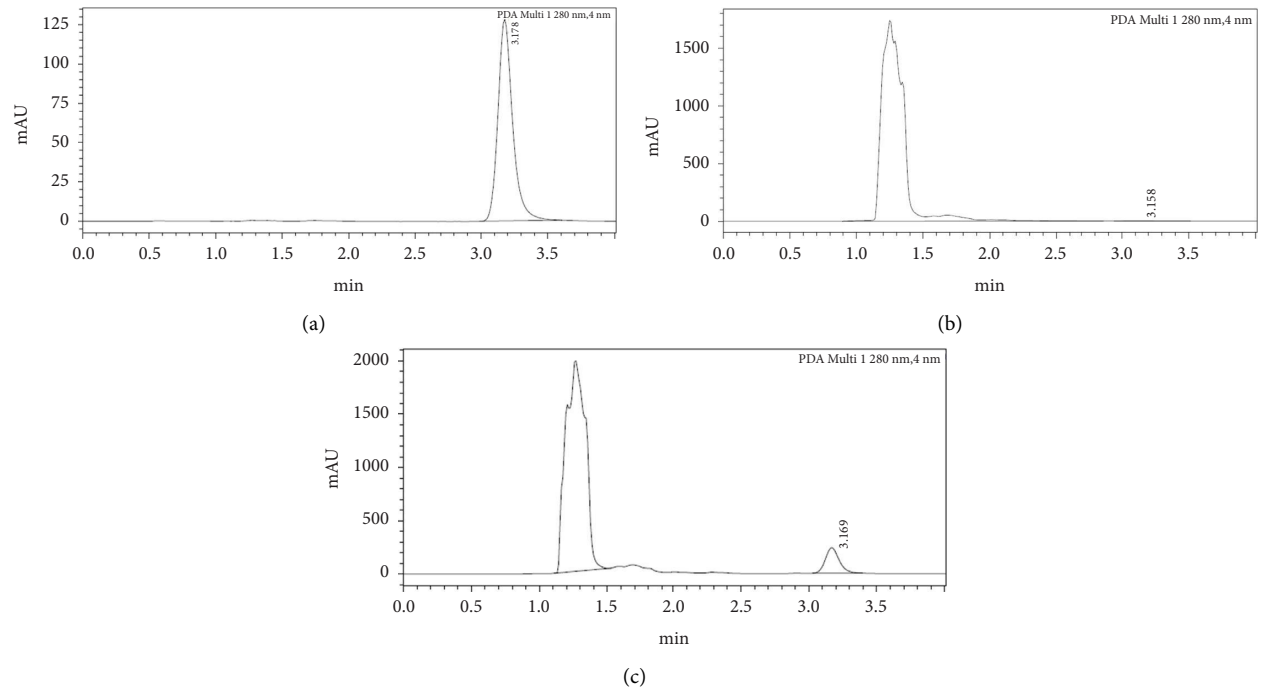

Figure 1: HPLC chromatograms of eugenol: (a) standard, (b) control, and (c) 1.0 mM SA treatment.

Difference (HSD) at $P < 0.05$. The mean values of three replicates were presented. The correlation between SA concentration, bioactive compounds, and antioxidant activity in the holy basil leaves was assessed using Pearson's correlation test, while principal component analysis (PCA) was carried out using JMP statistical software.

3. Results and Discussion

3.1. Effect of SA Concentration on Plant Growth. The results showed that SA foliar application at different concentrations was unable to improve plant growth. Plant height ranged from 40.43 ± 0.80 to 44.76 ± 1.93 cm, which was not significantly different among the treatments (Figure 2(a)). The highest and lowest shoot fresh weights of 38.43 ± 3.31 and 27.64 ± 0.74 g/plant were obtained in 0.1 and 2.5 mM SA treatments, respectively, but were not statistically differenced with the control (Figure 2(b)). Leaf fresh and dry weights of SA treatments revealed nonsignificant differences with those of control except the lower leaf fresh weight for the 1.0 and 2.5 mM SA treatments (Figures 2(b) and 2(c)).

SA, an endogenous regulator, may have an impact on plant growth and production [21]. It stimulates plant growth by changing the hormonal status and improving photosynthesis, transpiration, and stomatal conductance [6, 8]. The effect of exogenous SA on growth depends on various factors, such as plant species, developmental stages, and concentration. Plant growth of holy basil in the present study was not influenced by SA foliar application at different doses. Moreover, a single application might not be sufficient to stimulate mechanisms for plant growth. Similar results were reported by Damalas [22] who stated that under 100% field capacity, there were no significant differences between 200 ppm SA foliar application (applied once at 5-6 true leaves stage) and control for sweet basil shoot fresh weight, dry weight, or plant height. Jaafari and Hadavi [11] showed that foliar spray of SA three times (at 24, 34, and 44 days after emergence) at the concentrations of 2 and 4 mM did not affect the fresh weight and dry weight of sweet basil. In contrast, exogenous SA application increased plant growth of sweet basil [10, 23], marjoram [10], peppermint [8], and *Ammi visnaga* [15]. Elhindi et al. [23] showed that the application of 1.0 mM SA twice, first at the seedling stage with 2-3 true leaves and then 5 days after transplanting, significantly increased plant height, shoot fresh and dry weight, branch number, and leaf area of sweet basil. Likewise, Gharib [10] reported that 10^{-4} M SA foliar application at 75 and 82 days after sowing, with a repeat two weeks after the first and second cut, stimulated the growth of sweet basil and marjoram by enhancing photosynthesis and nutrient uptake. The 2.0 mM SA treatment resulted in improvement of the leaves and shoots in peppermint [8]. The application of similar concentrations of SA (2 mM) seven times increased the plant growth of *Ammi visnaga* under normal irrigation [15]. These findings indicate that the enhancement of plant growth by SA depends not only on its concentrations but also on the frequency of applications.

3.2. Effect of SA Concentrations on Bioactive Compounds. The yield of the dry extract ranged from 17.01 ± 3.47 to $20.81 \pm 2.50\%$, and there were no significant differences observed among the SA foliar applications at various concentrations (Figure 3).

SA foliar application at various concentrations affected the eugenol content in holy basil. The maximum eugenol content of $17{,}829.53 \pm 243.11$ μg/g dry extract occurred in 1.0 mM SA treatment, which increased 282.96 times over control. Eugenol content exhibited significant increases of

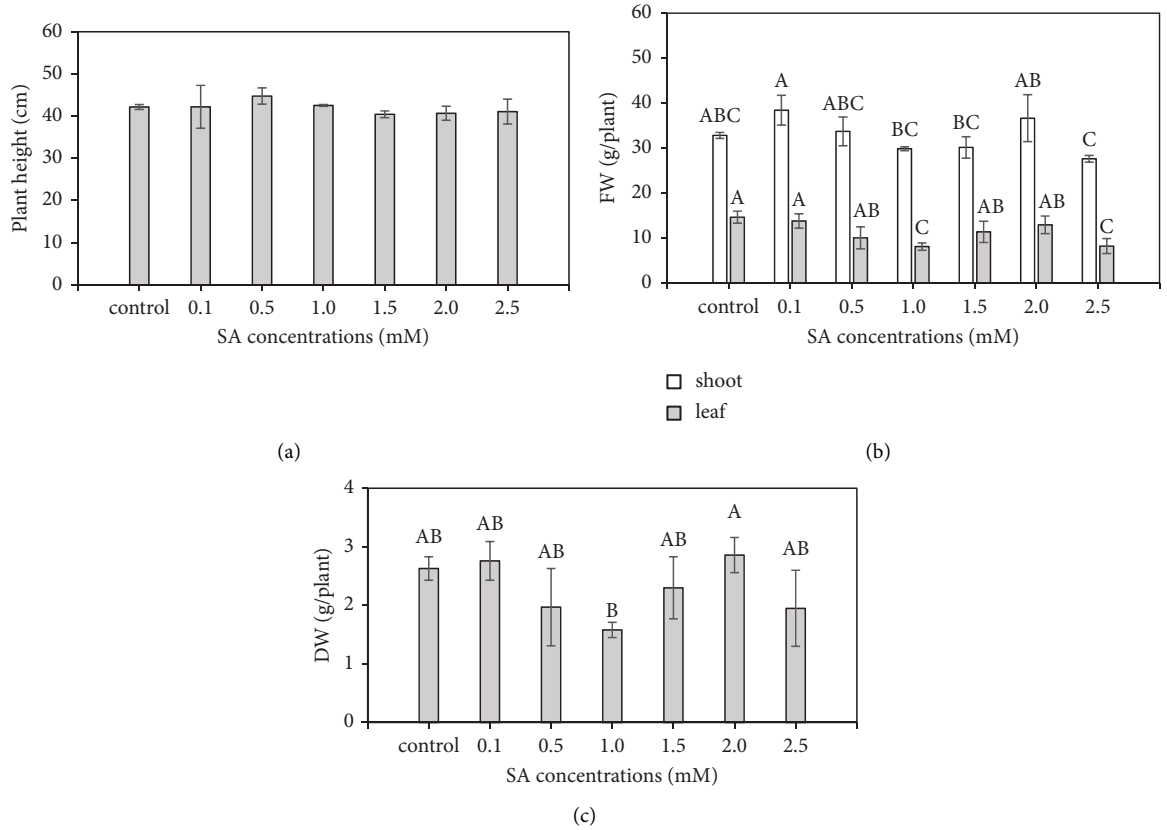

Figure 2: Effect of SA foliar application on (a) plant height, (b) shoot and leaf fresh weight (FW), and (c) leaf dry weight (DW) of holy basil. Data are means of three replicate samples and error bars indicate ± SD. The same uppercase letters indicate nonsignificant difference by Tukey's HSD (honestly significant difference) test at $P < 0.05$.

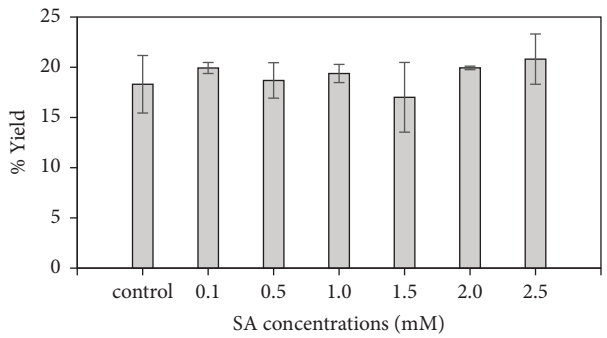

Figure 3: Effect of SA foliar application on yield of dry extract. Data are means of three replicate samples, and error bars indicate ± SD.

68.64, 182.02, and 78.21 times above control when applied SA at the concentrations of 0.1, 0.5, and 1.5 mM, respectively. Low eugenol contents of 201.09 ± 12.98 and 244.21 ± 12.75 μg/g dry extract were obtained in 2.0 and 2.5 mM SA treatments, respectively, which were not significantly different compared to the control (Figure 4(a)).

SA concentrations of 0.1, 0.5, 1.0, and 1.5 mM induced the accumulation of total phenolics. The highest content of total phenolics of 444.10 ± 2.80 mg GAE/g dry extract was observed in 1.0 mM SA treatment which was 1.76 time higher than control. While 2.0 and 2.5 mM SA treatments resulted in a lower total phenolic content of 209.01 ± 3.69 and 189.99 ± 1.35 mg GAE/g dry extract, respectively, than the control (Figure 4(b)).

The changes in total flavonoid contents under SA foliar application exhibited a similar trend as total phenolic contents. The maximum content of total flavonoids of 382.69 ± 6.49 mg QE/g dry extract occurred in 1.0 mM SA treatment which was 2.14 times above control. Total flavonoid contents of 0.1, 0.5, and 1.5 mM SA treatments showed higher values than those of the control while those of 2.0 and 2.5 mM SA treatments exhibited lower values of 140.42 ± 2.91 and 129.67 ± 5.97 mg QE/g dry extract, respectively, than those of the control (Figure 4(c)).

SA concentrations are crucial in inducing holy basil to produce bioactive compounds. In the present study, low SA concentrations at 0.1, 0.5, 1.0, and 1.5 mM demonstrated effective induction of eugenol and total phenolic and flavonoid accumulation. A maximum increase was observed at 1.0 mM SA treatment as the contents of eugenol, total phenolics, and total flavonoids were 282.96, 1.76, and 2.14 times, respectively, above control. On the contrary, high SA concentrations at 2.0 and 2.5 mM showed negative effects as the contents of those bioactive compounds were lower than those of control. These results indicated that SA stimulated bioactive compound accumulation in holy basil depending on its

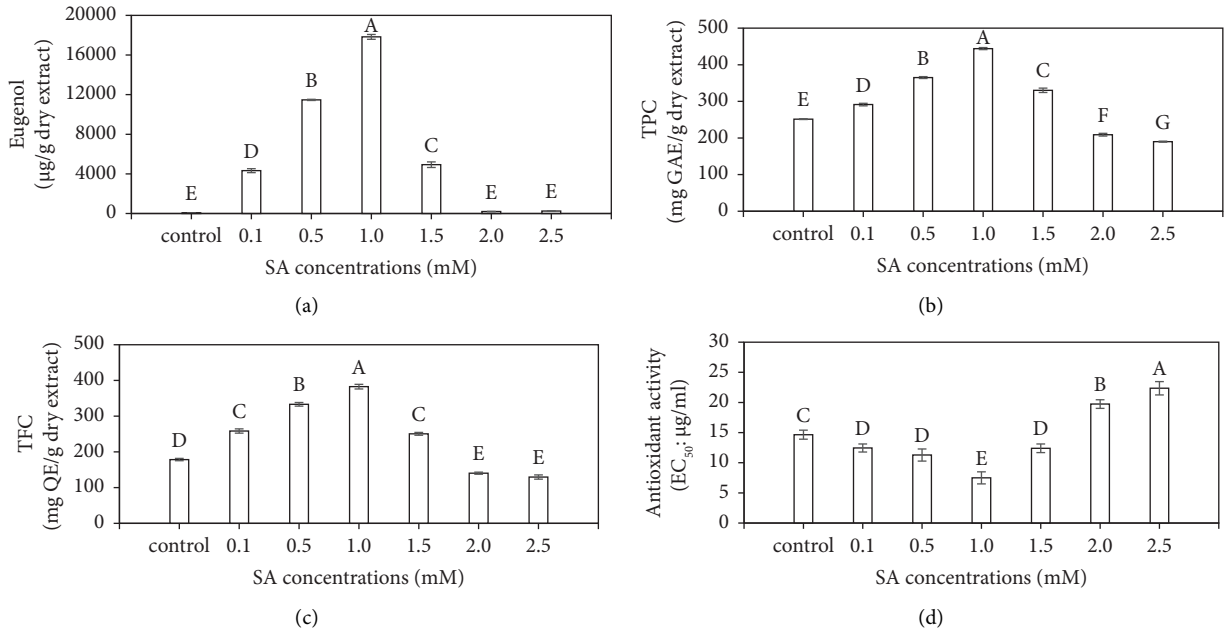

Figure 4: Effect of SA foliar application on (a) eugenol content, (b) total phenolic content (TPC), (c) total flavonoid content (TFC), and (d) antioxidant activity of holy basil. Data are means of three replicate samples and error bars indicate ± SD. The same uppercase letter indicates nonsignificant difference by Tukey's HSD (honestly significant difference) test at $P < 0.05$.

concentration. As a hormone-like substance of SA, high concentrations are typically harmful to physiological processes and can cause adverse effects, whereas appropriate SA concentrations regulate key enzymes like phenylalanine ammonia lyase and isochorismate synthase, which promote the formation of secondary metabolites and their subsequent storage in plant tissue [5]. The findings of this study were in agreement with those of earlier research, as exogenous SA application at low doses significantly induced the accumulation of bioactive compounds [8, 10, 11, 13]. Figueroa-Pérez et al. [8] reported that foliar application of 0.5 and 1.0 mM SA was more effective for enhancing total phenolic and flavonoid contents in peppermint than 2.0 mM SA. Essential oil content of peppermint was significantly increased at 150 mg/L SA treatment, but it was statistically decreased under increasing SA concentrations of 300 and 400 mg/L treatments [13]. Similar to sweet basil, the exogenous application of 2.0 mM SA resulted in a higher yield of essential oil compared to 4.0 mM SA [11]. Moreover for sweet basil, the higher eugenol level [10] and essential oil content [12] over control were obtained in foliar spray SA at 1.0 mM.

3.3. Effect of SA Concentrations on Antioxidant Activity. The strongest antioxidant activity with the lowest EC_{50} value of $7.49 \pm 1.00\ \mu$g/mL was achieved in 1.0 mM SA treatment. The EC_{50} value of BHT, the positive control, was $11.24 \pm 0.63\ \mu$g/mL. The 0.1, 0.5, and 1.5 mM SA treatments also revealed lower EC_{50} values than the control. On the other hand, the weak antioxidant activities occurred in 2.0 and 2.5 mM SA treatments, in which EC_{50} values of 19.73 ± 0.72 and $22.36 \pm 1.11\ \mu$g/mL, respectively, were higher than those of the control (Figure 4(d)).

Antioxidant activity measured by a DPPH radical scavenging assay is commonly used to determine the ability of the sample to provide hydrogen atoms. The response of antioxidant activity to exogenous SA concentrations exhibited the same trend as bioactive compounds. Low SA concentrations between 0.1 and 1.5 mM showed a significant decrease in EC_{50} values in comparison to the control. The 1.0 mM SA showed the most effective antioxidant activity as the lowest EC_{50} value occurred. These effects could contribute to the increase in bioactive compound accumulation in low SA concentration treatments. Eugenol, total phenolics, and total flavonoids are the major antioxidant compounds in holy basil [2]. These compounds revealed a strong negative correlation with EC_{50} values of antioxidant activity as shown in Table 1 ($R = -0.826$, $R = -0.940$, and $R = -0.922$, respectively, $P < 0.01$). This implied that a high content of these antioxidant compounds had the potential to scavenge free radicals. Antioxidant activities responded to SA doses were different among plant species. Exogenous SA at low doses, known for its potent antioxidant activity, has been documented in other plant species. The infusions prepared from peppermint leaves treated with 0.5 and 1.0 mM SA exhibited a greater capacity to inhibit DPPH radicals compared to those treated with 2.0 mM SA treatment [8].

3.4. Correlation Analysis and PCA of SA Concentration, Bioactive Compounds, and Antioxidant Activity. The SA concentration exhibited a moderate negative correlation with total flavonoid content, while showing a strong positive correlation with the EC_{50} value of antioxidant activity (Table 1). Moreover, the SA concentration displayed no

Table 1: Pearson's correlation coefficient between concentrations of SA, bioactive compounds, and antioxidant activity in the leaves of holy basil.

Parameter	Pearson's correlation coefficient				
	SA	Eugenol	TPC	TFC	AA
SA	1				
Eugenol	-0.258^{ns}	1			
TPC	-0.422^{ns}	0.954^{**}	1		
TFC	-0.462^{*}	0.959^{**}	0.983^{**}	1	
AA	0.630^{**}	-0.826^{**}	-0.940^{**}	-0.922^{**}	1

TPC: total phenolic content; TFC: total flavonoid content; AA: antioxidant activity. *Correlation is significant at $P < 0.05$, **correlation is significant at $P < 0.01$, and ns: not significant.

correlation with eugenol and total phenolic content. The results suggested that the application of exogenous SA at low concentrations resulted in increased levels of total flavonoids and greater antioxidant activity. However, it did not have any effect on eugenol and total phenolic content.

PCA was conducted to enhance the comprehension of correlations among the variables. The initial two principal components, PC1 and PC2, explained 97.9% of the total variation (Figure 5). PC1 was significantly influenced by all variables. Notably, the vectors representing SA concentration and antioxidant activity were opposite to those of eugenol, total phenolic, and total flavonoid contents on PC1, indicating an absence of direct association between bioactive compounds and SA concentration as well as antioxidant activity. All variables loaded positively on PC2, with antioxidant activity and total phenolic and flavonoid contents being less significant compared to SA concentration and eugenol. The samples with identical SA concentrations formed distinct clusters. Specifically, samples with SA concentrations of 0.1, 0.5, 1.0, and 1.5 mM exhibited positive loading on PC1, indicating higher contents of eugenol and total phenolic and total flavonoid compounds, coupled with lower levels of EC_{50} of antioxidant activity. Conversely, SA concentrations of 0, 2.0, and 2.5 mM exhibited negative loading on PC1, indicating lower content of bioactive compounds and higher levels of EC_{50} of antioxidant activity.

To accelerate bioactive compound accumulation, plants usually respond to the elicitor during a short duration after application. However, the appropriate preharvest application time inducing secondary metabolites differed in each plant species. For example, the 0.01 mM SA exogenous applied at 1 day before harvest revealed the highest total phenolic content in coriander [24]. In the case of Chinese kale, higher contents of vitamin C, total chlorophyll, and total phenolics occurred in 1.0 mM SA foliar sprayed at 6 days before harvest [25] while mustard foliar sprayed with 10^{-2} mM at 15 days before harvest resulted in an increase of total chlorophyll peroxidase and superoxide dismutase [26]. Therefore, we continuously studied the preharvest times of exogenous 1.0 mM SA application at 1, 2, 3, 4, and 5 days before harvest. It was found that SA foliar sprayed three days before harvest possessed the highest contents of bioactive compounds and antioxidant activity in holy basil (data not shown). Based on the current study, exogenous SA application could be a promising tool to enhance bioactivity in holy basil. The most effective method to improve the contents of eugenol, total phenolics, and total flavonoids as well as antioxidant activity was 1.0 mM SA foliar sprayed three days before harvest.

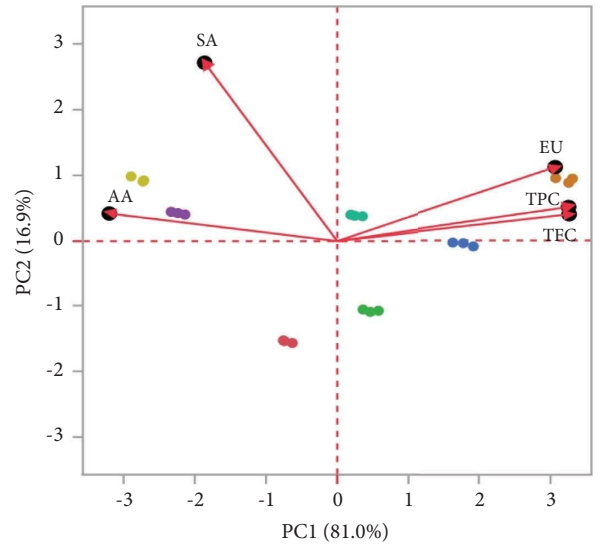

Figure 5: Biplot of principle component analysis of SA concentration, antioxidant activity (AA), and the content of eugenol (EU), total phenolic content (TPC), and total flavonoid content (TFC) of all samples. Color of symbols represents SA concentrations: red, 0 mM; light green, 0.1 mM; blue, 0.5 mM; brown, 1.0 mM; dark green, 1.5 mM; purple, 2.0 mM; yellow, 2.5 mM.

4. Conclusions

Exogenous SA at low concentrations of 0.1–1.5 mM enhanced the contents of eugenol, total phenolics, and total flavonoids in holy basil. The strong antioxidant activity was achieved when 0.1–1.5 mM SA was foliar sprayed. Foliar application of 1.0 mM SA was an effective method to produce enriched bioactivity in holy basil.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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